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Structure–Activity Relationships of Estrogens

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The last 50 years has seen an exponential rise in the published reports about estrogen action. The model to describe the early events in the mechanism of action of estrogens via the estrogen receptor is updated in this paper to incorporate some of the recent data on the subcellular localization of the receptor. New evidence suggests that the receptor is a nuclear protein, so it appears that estrogens must first diffuse into the nuclear compartment to initiate estrogen action via the receptor complex. This review traces the development of potent estrogenic compounds by the study of their structure–activity relationships. Studies of structure–activity relationships *in vivo* using Allen Doisy or 3-day uterine weight tests can provide much valuable information, but the assays suffer from the complex problems of pharmacokinetics and metaboiic transformation. Studies *in vitro* using primary cultures of rat pituitary or uterine cells to assay the ability of a compound to induce prolactin synthesis or progesterone receptor synthesis, respectively, can provide essential information about the structural requirements for a compound to produce estrogenic effects. Nevertheless, it should be pointed out that studies *in vivo* are required to determine whether a compound is metabolically activated to an estrogen. Estrogen receptor binding models are presented to describe the changes in a molecule that will predict high affinity for the ligand and agonist, partial agonist and antagonist properties of the ligand–receptor complex. Most estrogenic pesticides and phytoestrogens comform to the predictions of the estrogen receptor binding model.

Introduction

It is now more than half a century since the first compound, with known chemical structure, was shown to produce an estrogenic effects in animals (*1*). Since that time, thousands of compounds have been screened for estrogenic activity. In the past quarter century the early events involved in the molecular mechanism of action of estrogens in their target tissues (e.g., vagina, uterus, pituitary gland or breast), via the estrogen receptor, have been described (*2–5*). The aim of this paper is to review the evolution of structure–activity relationship studies and suggest estrogen receptor models to predict biological activity. A brief survey of the structure activity relationships of phytoestrogens, pesticides and antiestrogens will be used to demonstrate the recurrent molecular features necessary for a compound to bind to the estrogen receptor and/or to produce a conformational change in the receptor complex that will initiate agonist or antagonist actions.

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Historical Perspective: The First 25 Years

The pioneering studies by Sir Charles Dodds laid the foundation for all the subsequent research on the structure activity relationships of nonsteroidal estrogens. The 1930s saw a remarkable expansion of knowledge that culminated in the description of the optimal structural requirements in a simple molecule to produce estrogen action. The first compound of known structure (1-keto-1,2,3,4-tetrahydrophenanthrene) (Fig. 1, compound 2) to be found to have estrogenic activity (*1*) was tested because of its structural similarity to the presumed structure of ketohydroxy estrin (Fig. 1, compound 1). As it turned out, the structure of the natural steroid (estrone) was incorrect, but this did not matter; the fact that nonsteroidal compounds can exhibit estrogenic properties was established. A phenanthrene nucleus was later found to be unnecessary for estrogenic activity (*6*). Simple bisphenolic compounds are active (Fig. 1, compounds 3–6) and, as will be seen later in the review, this is a recurrent feature of many nonsteroidal estrogens. The finding that hydroxystilbenes (Fig. 1, compounds 7–9) possess potent estrogenic activity provided a valuable clue that stimulated a systematic investiga-

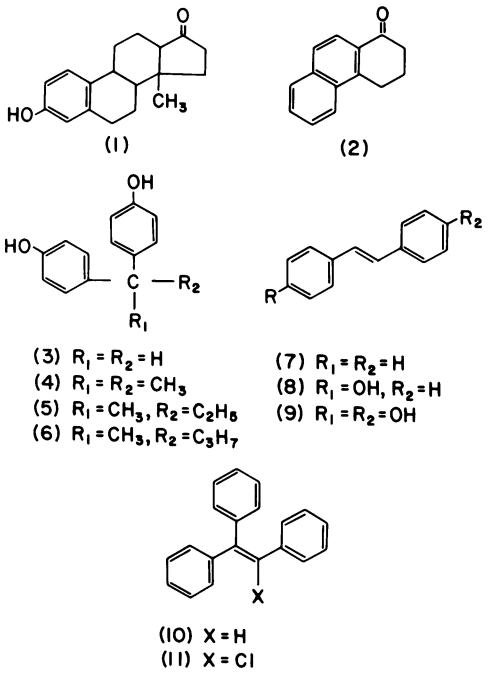


FIGURE 1. Formulae of compounds found, in the 1930s, to have estrogenic activity *in vivo*. Compound 1 was believed to be the molecular structure of ketohydroxyestrin (estrone). This is now known to be incorrect.

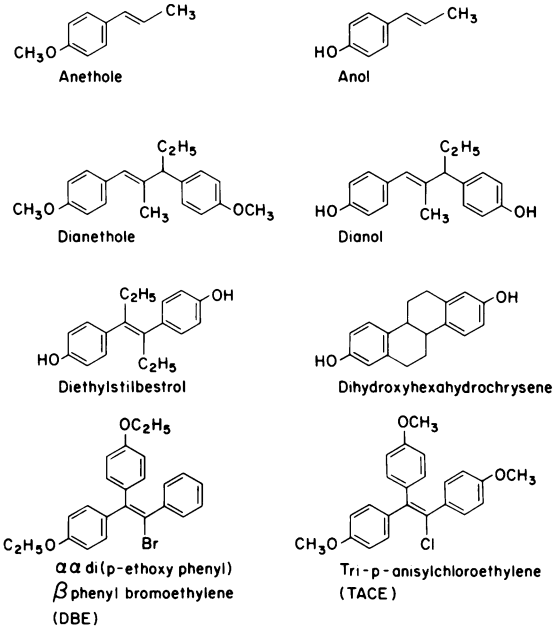


FIGURE 2. Formulae of nonsteroidal compounds with estrogenic (or suspected) activity *in vivo*.

tion of analogs to optimize the potency. At this time, an interesting side issue occurred that deserves comment, as it illustrates how parallel research endeavors can eventually reach the same conclusions. Anol, a simple phenol derived from anethole (Fig. 2), was reported to possess extremely potent estrogenic activity with 1 μg capable of inducing estrus in all rats (*7*). These results were not confirmed with different preparations of anol (*8,9*), but it was found that dimerization of anol to dianol (Fig. 2) can occur and this impurity, which was known to have potent estrogenic (*10*) properties, was the compound responsible for the controversy (*11*). At this time, Dodds reported (*12*) that diethyl substitution at the ethylenic bond of stilbestrol (Fig. 2) produces an extremely potent estrogen (*12,13*); other substitutions produce less active compounds (*14*). The structural similarity between diethylstilbestrol and estradiol (the formula was established by 1938) was noted (*12*), but an attempt to mimic the rigid steroid structure by the synthesis of dihydroxyhexahydrochrysene (Fig. 2) demonstrated a drop in estrogenic potency. Dihydroxyhexahydrochrysene is approximately 1/2000 as potent as diethylstilbestrol.

There was considerable interest in the development of a long-acting synthetic estrogen because of the potential for clinical application. The duration of action of diethylstilbestrol can be increased dramatically by esterification of the phenolic groups (*15*). A 10-μg dose of diethylstilbestrol dipropionate can produce estrus for more than 50 days, while the phenol at the same dose is active for only 5 days. The simple hydrocarbon triphenylethylene is a weakly active estrogen (*16*), but 10 mg can produce vaginal cornification in mice for up to 9 weeks. Replacement of the free ethylenic hydrogen with chlorine (Fig. 1) increases the potency and duration of action by subcutaneous administration (*17*), but when administered orally, triphenylchloroethylene has a similar duration of action as diethylstilbestrol or estradiol benzoate. In the search for orally active agents, Robson and Schonberg (*18*) showed that DBE (Fig. 2) was very effective by the oral route. The long duration of action is related to depot formation in body fat (*19*), but DBE did not reach clinical trial. The related compound, trianisylchloroethylene (TACE) is, however, available clinically as a long-acting estrogen (Fig. 2). TACE is stored in body fat for prolonged periods (*20–22*).

The first 25 years established many of the important structural features that govern the potency and duration of action of estrogens. Investigations for the next 25 years focused upon the subcellular mechanism of estrogen action.

Estrogen Action

The reason for the target site specificity of the estrogens remained obscure until the synthesis of tritium-labeled compounds with high specific activity. The synthesis of [^{3}H]hexestrol (reduction of diethylstilbestrol with tritium and a palladium catalyst) by Glascock working with Sir Charles Dodds (*23*) and the subsequent observation that there was binding of hexestrol in the uterus, vagina, mammary glands and pituitary gland of immature female goats and sheep (*24*) provided the first evidence for the target tissue localization of estrogens. The subsequent fundamental study by Jensen and Ja-

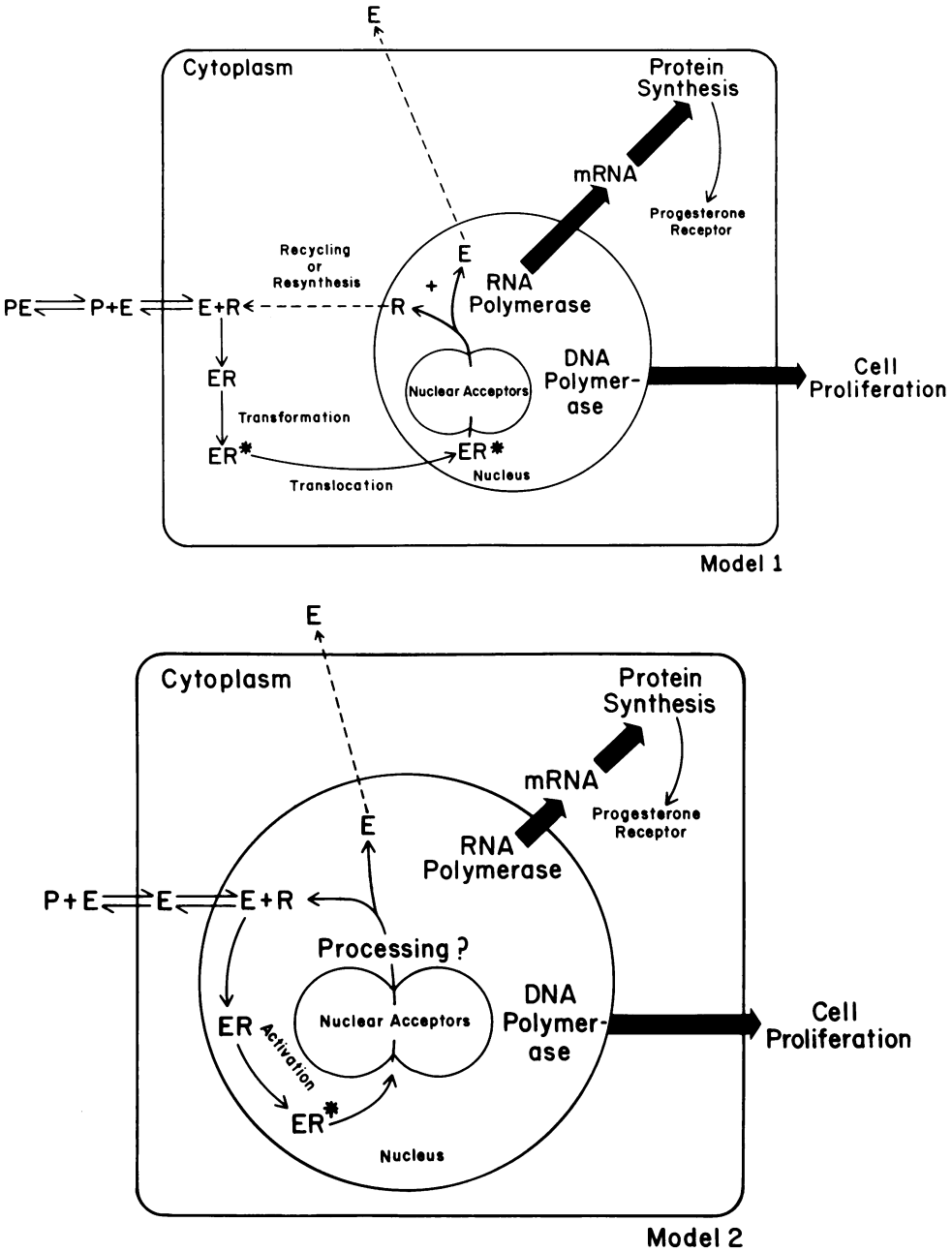


FIGURE 3. Subcellular models of estrogen action. Estrogen (E) dissociates from plasma proteins (P) and diffuses into the target cell. In Model 1 the receptor (R) is located in the cytoplasm and in Model 2 the receptor is in the nuclear compartment. In both models the receptor complex is activated before initiating protein synthesis and finally cell division. The fate of the receptor is at present unknown; it may be recycled, destroyed (processed) or resynthesized.

cobson (*2*) of the distribution and binding of [^{3}H]estradiol in the immature rat demonstrated that estradiol selectively binds to, and is retained by, the uterus, vagina, and pituitary gland. These studies suggested that there is a specific receptor for estradiol in its target tissues. The biochemical identification of an estrogen binding protein in the immature rat uterus and the observation that [^{3}H]estradiol becomes located in the nucleus of the cell provided a model to describe the initiation of estrogen-stimulated events. The evidence for an estrogen receptor system has been reviewed (*3*,*4*), and the model illustrated in Figure 3 has been proposed. The estrogen dissociates from plasma proteins and readily diffuses into the cell. The cytoplasmic estrogen receptor binds the ligand and the resulting receptor complex is activated before translocation to the nucleus. Interaction with nuclear acceptors results in the activation of RNA and DNA polymerases to initiate subsequent protein synthesis and cell proliferation, respectively. There are, however, an increasing number of observations that are

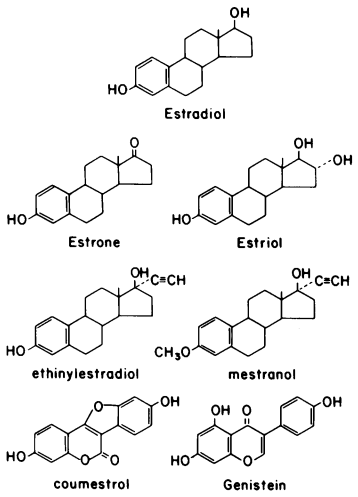


FIGURE 4. Formulae of some steroidal estrogens and the phytoestrogens coumestrol and genistein.

inconsistent with the classical two-step hypothesis. These reports have been reviewed (*5*). Two recent innovative approaches to the question of the subcellular localization of unoccupied estrogen receptor deserve comment. Monoclonal antibodies raised to the estrogen receptor are being used as tags for immunohistochemical studies. The antibody is linked to a peroxidase enzyme system to visualize the receptor, which appears to be located exclusively in the nuclear compartment, even in the absence of estrogen (*25*). The other approach has been to enucleate estrogen receptor containing GH3 rat pituitary tumor cells with cytochalasin B. Unoccupied receptors are observed in nucleoplasts rather than cytoplasts (*26*). Although it is possible that these studies are generating artifactual results, the simplified model of estrogen action illustrated in Figure 3 should also be considered to represent subcellular events *in vivo*.

Testing Methods for Estrogens

The Allen-Doisy test (*27*) depends upon the induction of vaginal cornification in castrate animals 60–80 hr after the subcutaneous administration of estrogens. A colony of animals is ovariectomized and used for assays two weeks later. To maintain the sensitivity of the colony and retard atrophy of the uterus and vagina (*28*), the animals are primed with 1 μg estradiol (SC) every 6 weeks. The animals are not used for 2 weeks following either priming or experimental use. However, it is often wise to screen the animals by the vaginal smear technique to check for incomplete ovariectomy or test compounds with prolonged biological activity.

Direct administration of estrogens into the vagina increases the sensitivity of the Allen-Doisy test and cornification occurs earlier since the response is not dependent upon distribution and metabolism (*29,30*). Emmens (*29–32*) has assayed and evaluated the structural derivatives of stilbene and triphenylethylene by both intravaginal and systemic Allen-Doisy tests. This early

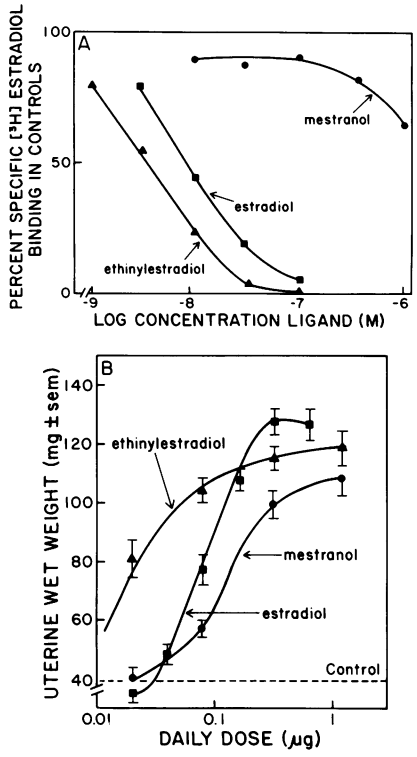


FIGURE 5. Receptor binding and uterotrophic activity of estradiol, ethinylestradiol, and mestranol: (*A*) inhibition of the binding of [^{3}H]estradiol to rat uterine estrogen receptors *in vitro* by ethinyl estradiol, estradiol, and mestranol; (*B*) uterotrophic activity of ethinyl estradiol, estradiol, and mestranol in the 3-day immature rat uterine wet weight test. Data adapted from Allen et al. (*49*).

work accurately established the relative potency of the test compounds.

Martin and Claringbold (*33*) developed the intravaginal assay to study the early events of estrogen stimulation by using the increase in vaginal mitoses and vaginal epithelium thickening as measures of the estrogenic response. Martin (*34*) further showed that the reduction of 2,3,5-triphenyltetrazolium chloride to formazan in epithelial cells of the vagina following the local application of estrogens could form the basis of a sensitive assay procedure for early estrogenic events.

The increase in uterine weight of young castrate rats was used to determine systemic estrogen activity by Bülbring and Burn (*35*). The preparation of castrate animals has been found to be an unnecessary step, and immature rats or mice are usually used (*36,37*). Estrogens induce a rapid early imbibition of water by the uterus, and this effect has been used in the 6-hr assay of estrogens by Astwood (*38*). However, this technique cannot distinguish between full estrogens and partial agonists and also suffers from differences in the release of test compounds from the injection site which will ultimately affect the time course of the uterine response. Most assays utilize a 3-day injection technique to stimulate full uterine growth (*39*).

Potential estrogenic activity can be inferred for a compound by its ability to inhibit the binding of [^{3}H]estradiol

to its target tissues *in vivo* (*40,41*). However, many nonsteroidal antiestrogens produce the same effect (*42,43*) so this effect cannot be assumed to predict biological activity. Similarly, the ability of a compound to inhibit the binding of [^{3}H]estradiol to estrogen receptors *in vitro* suggests a potential mechanism of action via the estrogen receptor but again this cannot predict biological activity, i.e., agonist or antagonist actions (*44*).

The evaluation of a compound to inhibit the binding of [^{3}H]estradiol to the estrogen receptor *in vitro* (*45,46*) must be used in combination with an evaluation of estrogenic properties *in vivo*. This approach will avoid the misclassification of a compound as inactive based upon a low potential to bind to receptors *in vitro*, only to find it is converted to a potent estrogen *in vivo*. Mestranol (Fig. 4) has a very low affinity for estrogen receptors *in vitro*, but *in vivo* it is rapidly demethylated to the potent estrogen ethinyl estradiol (*47,48*). These principles are illustrated in Figure 5. Demethylation of methyl ethers to phenols is a primary route of metabolic activation for several phytoestrogens and insecticides.

While studies *in vivo* are necessary to evaluate the potential for the metabolic activation of a compound, studies of structure–activity relationships require precise assays *in vitro* where the concerns of metabolic transformation can be minimized. We are currently using two assay techniques *in vitro*: a prolactin synthesis assay (*50,51*) and progesterone receptor synthesis assay (*52*), in combination with a determination of the relative binding affinity of a compound for the estrogen receptor. Representative results are illustrated in Figure 6 for the estrogens estradiol, estrone and estriol and compared with the estrogen antagonist 4-hydroxytamoxifen. This antiestrogen has a high affinity for the estrogen receptor (*53*). Several points can be made about the results of these studies. The assays *in vitro* avoid the problems of pharmacokinetics encountered with the administration of short-acting estrogens like estriol in uterine weight tests (*54*). Potency appears to be low in traditional assays *in vivo* because the steroid is cleared before a biological effect can be produced. Sustained release preparations that are implanted into animals demonstrate high biological activity for estriol (*54*). This principle is readily illustrated in the assays *in vitro* where cells are continuously exposed to ligands to initiate estrogen-stimulated prolactin (Fig. 6*B*) or progesterone (Fig. 6*C*) receptor synthesis. Clearly polyphenolic estrogens in the environment will be rapidly excreted following single daily administration to immature rats or mice. As a result, the compounds may produce no biological effects *in vivo* but may be shown to have biological effect in the assay *in vitro*. These assays *in vitro*

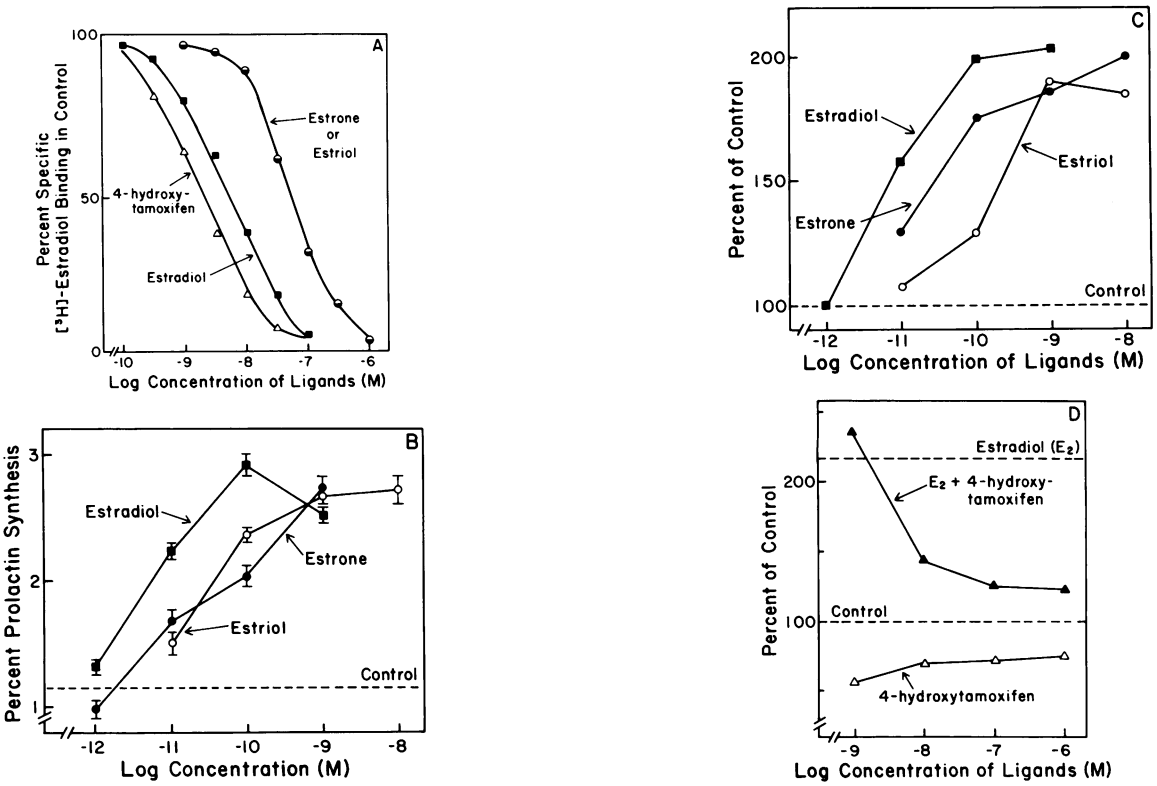


FIGURE 6. Receptor binding and biological activity *in vitro* of estradiol, estrone, estriol, and 4-hydroxytamoxifen: (*A*) inhibition of the binding of [^{3}H]estradiol to rat uterine estrogen receptors *in vitro* by 4-hydroxytamoxifen, estradiol, estrone, and estriol; (*B*) increase in the percent prolactin synthesis produced by the culture of dispersed cells from immature rat pituitary glands with increasing concentrations of estradiol, estriol, and estrone; (*C*) percent increase (over control) of progesterone receptor in cultures of dispersed cells from immature rat uteri with increasing concentrations of estradiol, estriol, and estrone; (*D*) percent decrease of estradiol (1 nM)-stimulated progesterone receptor in cultures of dispersed cells from immature rat uteri produced by increasing concentrations of 4-hydroxytamoxifen.

mimic continual exposure of an organ to a particular compound.

The result with the antiestrogen 4-hydroxytamoxifen (Fig. 6*D*) is of interest because it is a complete estrogen antagonist and does not stimulate either progesterone receptor or prolactin synthesis *in vitro* (*44,51*). 4-Hydroxytamoxifen stimulates progesterone receptor synthesis in the rat uterus *in vivo* (*55,56*). The pharmacological properties of antiestrogens *in vitro* have permitted the development of drug receptor models of estrogen and antiestrogen. These will be considered in the final section following a brief survey of the structure–activity relationships of phytoestrogens and selected estrogenic pesticides.

Phytoestrogens

Plants (*57*) and fungal contaminants of stored grain (*58*) contain estrogens that are suspected of contributing to infertility problems in farm animals. There is also the possibility that phytoestrogens in the diet can contribute to the growth of hormone-dependent breast tumors in postmenopausal patients (*59*). Other than the potentially harmful effects of estrogens, they are apparently important to improve the quality of meat. Grazing animals in areas that are rich in phytoestrogens could provide economic advantages.

There are three major chemical types of phytoestrogens in plants: flavones, isoflavones and coumestans. The formula of two estrogenic compounds, genistein (isoflavone) and coumestrol (coumestan), are usually depicted as shown in Figure 4. However, for discussion purposes the structures of a range of phytoestrogens have been redrawn in a novel fashion to compare biological potency–receptor binding and structure (Fig. 7).

The isoflavone derivatives have attracted much attention as estrogenic compounds in clover. The early structure–activity relationships studies were reviewed by Bradbury and White (*60*). Genistein is the most active estrogen of this group with the highest binding affinity (RBA 0.9) for the estrogen receptor (*61*). The methoxy derivative, Biochanin A, does not bind to the estrogen receptor, but is estrogenic *in vivo* (*61,62*) Similarly, diadzein has a higher binding affinity for the estrogen receptor than the methoxy derivative, formononetin; both are weak estrogens *in vivo* (*61,62*). (c.f., ethinylestradiol and mestranol). The metabolism of formonentin and Biochanin A by micro/organisms in the sheep rumen has been described (*63*); however, diadzein is further reduced to equol, a weak estrogen (*64*) that can be crystallized from urine.

Recently, a novel compound, enterolactone (HPMF), has been isolated from human urine (*65,66*) (Fig. 8). No biological properties or function have been ascribed for enterolactone, but it appears to be a product of microbial metabolic transformation in the intestine (*67,68*). In testing this compound we detected no estrogenic properties in a 3-day Rubin test using immature female rats and no ability to inhibit [^{3}H]estradiol binding to uterine

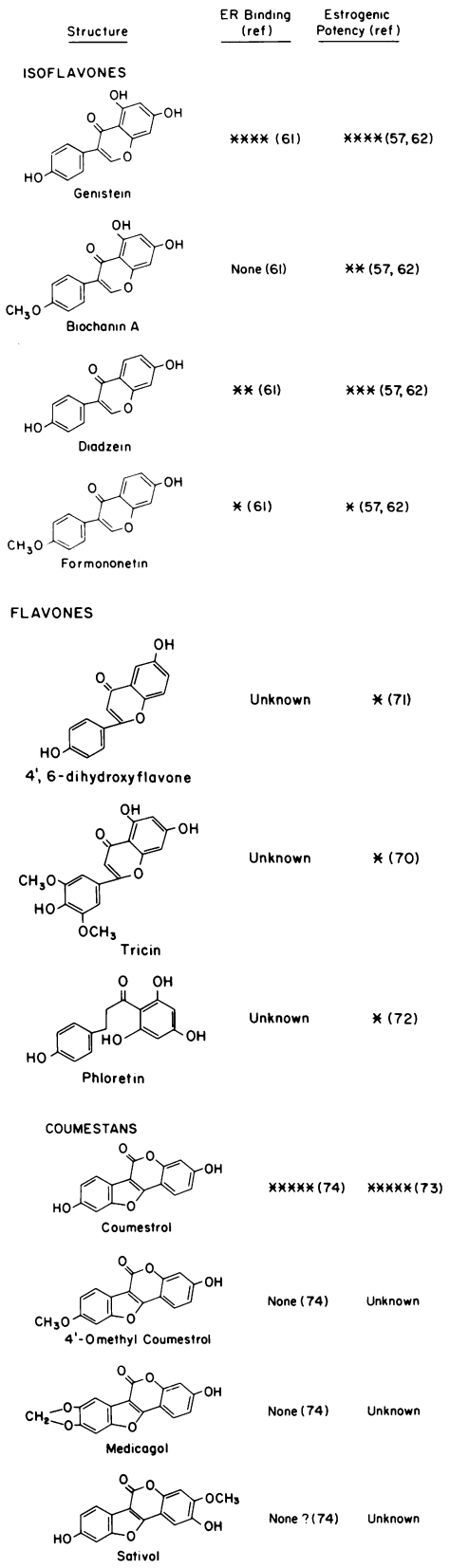


FIGURE 7. Structures, estrogenic potency (relative estimates based on coumestrol) and binding affinity for the estrogen receptor (relative estimates based on coumestrol) for 3 classes of phytoestrogens.

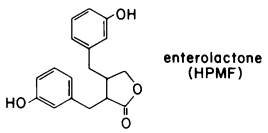


FIGURE 8. Formula of enterolactone which has also been referred to as HPMF (*65*).

estrogen receptors. However, we made two interesting observations that might have clinical implications. The nonsteroidal antiestrogen, tamoxifen, inhibits estradiol-stimulated prolactin synthesis by immature rat pituitary cells *in vitro* (Fig. 9*A*). HPMF does not affect estradiol-stimulated prolactin synthesis, but is significantly estrogenic at 10 μM (Fig. 9*A*). In another test that uses growth of MCF7 breast cancer cells as an endpoint, tamoxifen alone inhibits the growth of the cells, but this can readily be reversed with estradiol (*69*). HPMF (10 μM) can also reverse the inhibitory effect of tamoxifen on the growth of MCF7 cells (Fig. 9*B*). Clearly, high circulating levels of HPMF might be detrimental to patients undergoing antiestrogen therapy for breast cancer.

The flavones are very weak estrogens. The methoxyflavone, tricin, is a constituent of alfalfa (*70*) and very weakly estrogenic in the mouse. Several flavones (liqueritigenin, naringenin and hesperitin) have been shown to be inactive as estrogens in the immature rat; however, 4′,6-dihydroxyflavone (Fig. 7) is an active estrogen by subcutaneous administration but not orally (*71*). It is postulated that 4′,6-hydroxyflavone is converted to the ring opened chalconoid by alkali in the intestine. [N.B. The authors, Wenzel and Rosenberg (*71*), mistakenly state in their summary that 4′,6-dihydroxyflavone is only active orally and not subcutaneously as their data show.] However, a chalconoid, phloretin (Fig. 7), has been shown (*72*) to have estrogenic properties in immature mice if administered subcutaneously for 10 days; therefore, conversion of flavones to chalconoids cannot completely explain their lack of estrogenicity following oral administration.

Coumesterol (Fig. 7) is the most potent of the estrogens in forage crops (*73*) and, consistent with this observation, has a higher binding affinity for the estrogen receptor than genistein (*61,74*). As previously discussed for genistein, conversion of coumesterol to its methoxy derivatives prevents binding to the estrogen receptor (*74*). Medicagol does not bind to the estrogen receptor (*74*), which is consistent with the receptor binding model that appears to depend upon a phenolic group in the 4′ position of isoflavones and the 12 positions of coumestans. In contrast, sativol does not inhibit [^{3}H]estradiol binding (*74*). This seems to be anomalous, because the 12-hydroxyl is free and the 7-hydroxyl is methylated; however, the 6-phenol might be responsible for this effect.

The Fusarium fungus produces an estrogenic macrolide, zearalenone, which has been shown to have low binding affinity for estrogen receptors from rat uterus

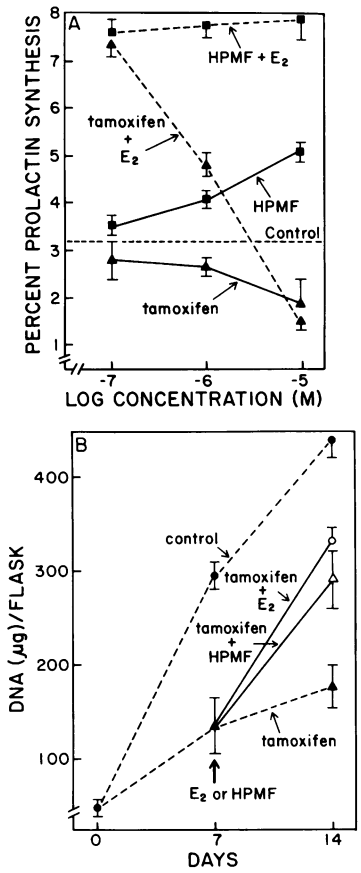
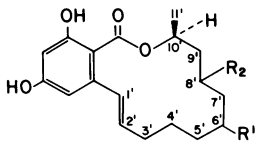


FIGURE 9. Biological activity of HPMF: (*A*) Inhibition of estradiol (1 nM)-stimulated prolactin synthesis by tamoxifen. HPMF did not inhibit estradiol-stimulated prolactin synthesis but 10 μM stimulated prolactin synthesis in immature rat pituitary cells in culture. (*B*) Inhibition of the growth of MCF7 breast cancer cells by tamoxifen (1 μM). Estradiol (10 nM) or HPMF (10 μM) reversed the effect of tamoxifen on cell growth.



NAME	C_1'-C_2'	R_1	R_2
trans zearalenone	trans	=O	H
zearalenol	trans	—OH	H
zearalenol	H_2-H_2	—OH	H
8′ hydroxyzearalenone	trans	=O	—OH
6′ aminozearalenone	trans	—NH_2	H

FIGURE 10. Structural derivatives of zearalenone.

(*75*), mammary gland (*76*), and the MCF-7 human breast cancer cell line (*59*). The structural requirements for receptor binding have been described (*75*) (Fig. 10). The *cis* and *trans* isomeric forms of zearalenone, zearalenol, and zearalanol all have a similar, low binding affinity for estrogen receptors. The 8′-hydroxyzearalenone and 6′-aminozearalene are inactive *in vivo* and *in vitro*. It is not, however, possible to state with assurance that the 4-phenolic hydroxy is important for receptor binding.

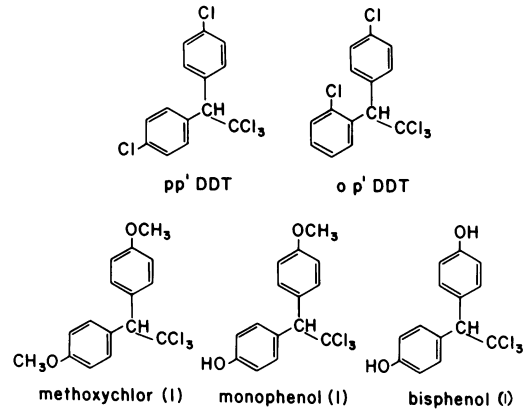


FIGURE 11. Formulae of some polychlorinated pesticides and the hydroxy metabolites of methoxychlor.

Pesticides

The chlorinated pesticides are known to alter the reproductive capacity of animals by an interaction with estrogen target tissues (*77–79*). The *o,p'* isomer of DDT (*80*), methoxychlor (*81*), and kepone (*82*) all have estrogenic activity in rat uterine weight assays. *p,p'*-DDT (Fig. 11) has an extremely low affinity for the estrogen receptor, but the *o,p'* isomer of DDT is activity and will inhibit the binding of [^{3}H]estradiol to estrogen receptors *in vivo* (*83*) and *in vitro* (*80,84*). The related compound, methoxychlor (Fig. 11), is estrogenic in the rat; however, its mono- and didemethylated metabolites, as would be expected from the structure (c.f. the early work of Dodds, Fig. 1), are capable of inhibiting the binding of [^{3}H]estradiol to its receptor *in vivo* (*85*) and *in vitro* (*86,87*).

Structure-Activity Studies *in Vivo*: Correlation with Estrogen Receptor Binding

There are many published reports on the structure-activity relationships of steroidal and nonsteroidal estrogens. For clarity, therefore, only select studies will be considered to illustrate relevant points. Dorfman and Kincl (*88*) used a 3-day immature mouse uterine weight test to determine the uterotrophic activity of 3- and/or 17-deoxy derivatives of estrone and estradiol and selected alkyl derivatives. The enhanced oral activity of the 17 α-ethinyl derivative of estradiol was confirmed, but in general other alkyl substitutions on the steroid nucleus were found to be a disadvantage. Using estrone as the standard, substitution with methyl at C_2, alkyl at C_4 or methylene at C_{16} all dramatically decreased estrogenic activity (Fig. 12). Estra-1,3,5(10)-triene has very low potency as an estrogen (Fig. 12). Potency is improved by substitution at C_{17} with either a ketone or hydroxyl; however, a phenolic hydroxyl at C_3 is the most important substitution (Fig. 12). This is consistent with the subsequent observation (*89*) that the 3-phenolic hydroxyl is the most important substitution for binding to the estrogen receptor.

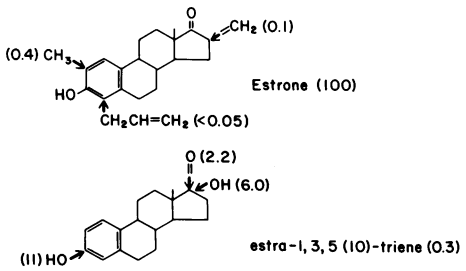


FIGURE 12. Structure–activity relationships of estrone. The structure–activity relationships of steroids in the 3-day immature mouse uterine weight test using estrone as the standard. The relative potencies are shown in parentheses using estrone equivalent to 100. Data adapted from Dorfman and Kincl (*88*).

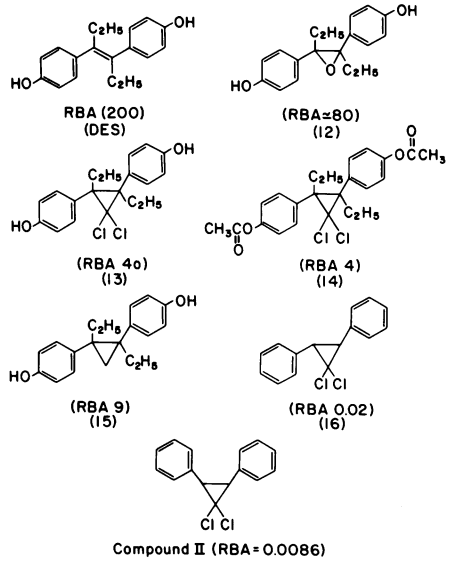


FIGURE 13. Relative binding affinities (RBAs) of several cyclopropyl analogs of stilbene and stilbenediol. Data adapted from Pento et al. (*90*).

Diethylstilbestrol has a high binding affinity for the estrogen receptor (*45*). A series of interesting cyclopropane analogs has recently been prepared (*90*) and their biological properties assessed. The relative binding affinities of several compounds are shown in Figure 13. The cyclopropyl analog (compound 15) most closely related to DES has low binding affinity (RBA 9) for the receptor, whereas a three-membered oxygen containing ring was less detrimental (RBA = 80) (compound 12). Substitution of the cyclopropyl derivative with two chlorines increased the RBA (compound 13), and, as previously observed, masking the phenols with acetyl group decreased the RBA (compound 14). An interesting biological observation with these compounds is that they are all estrogens of different potency except compound II, which is reported (*90*) to be a weak anties-

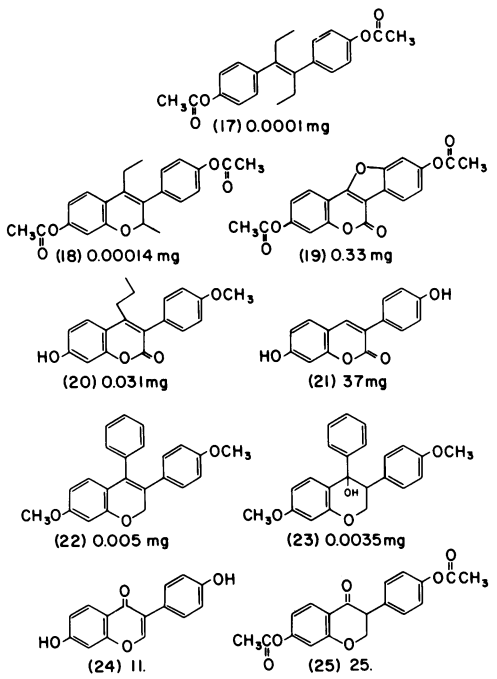


FIGURE 14. Structure–activity relationships of nonsteroidal estrogens related to phytoestrogens. The dose necessary to produce an increase of immature mouse uterine wet weight to 20 mg from a control value of 10 mg is shown for each compound (oral administration). Data adapted from Micheli et al. (*92*).

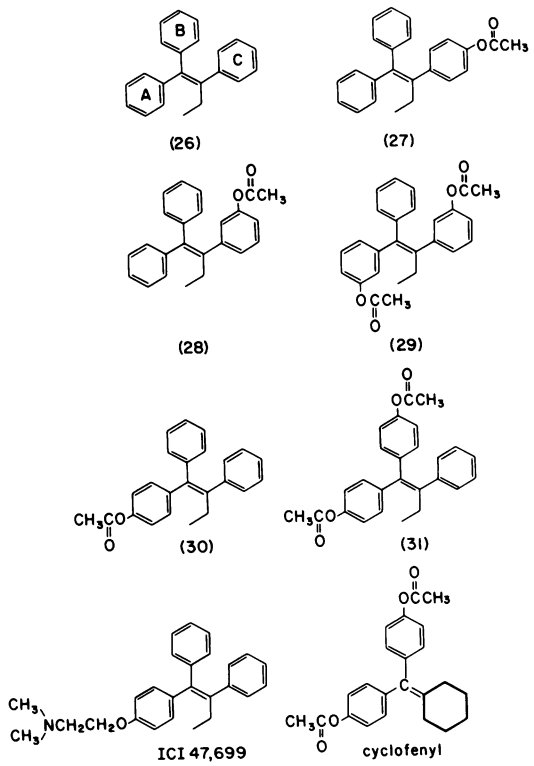


FIGURE 15. Nonsteroidal estrogens.

trogen in the mouse. There are no published reports of the structure–activity relationships of this *cis* cyclopropyl derivative, but it is possible that potency, and possibly effectiveness as an antiestrogen, could be improved by *para* substitutions with hydroxyl and an alkylaminoethoxyside chain [this is a recurrent feature of nonsteroidal antiestrogens (*91*)].

The structure–activity relationship of the plant phenolics has been compared with synthetic estrogens (*92*). In Figure 14 the potency of various compounds was determined as the minimum oral dose level required to produce an immature mouse uterine weight of 20 mg (control = 10 mg). The structural similarities between diethylstilbestrol and the test compounds is emphasized. As might be expected, diacetylation does not affect the potency of orally administered diethylstilbestrol. The Δ^3-isoflavens (compound 18) is equivalent is potency to compound 17. However, there is a dramatic decrease in potency with compound 22 which is related to the triphenylethylene-type of long-acting estrogens previously described (c.f. Fig. 2). It is interesting to note that the isoflavan-4-ol (compound 23) is slightly more active that its dehydrated derivative, compound 22. The coumarin derivatives (compounds 19-21) show a broad range of potencies. The low potency of compound 21 is dramatically improved by creating an oxygen bridge to form coumestrol (comparative dose level 0.25 mg). Acetylation of coumestrol to form compound 19 does not significantly decrease activity. Substitution of compound 21 with an *n*-propyl group increases potency from 37 mg to 0.1 mg, but introduction of a single methoxy group improves potency still further (compound 20) to 0.031 mg. This may be a pharmacokinetic effect to increase the lipophylicity and increase the biological half-life by preventing rapid conjugation. The isoflavanone, daidzein (compound 24), has a low potency of the same order as the isoflavanone compound 25.

Substituted triphenylethylene derivatives have been studied extensively *in vivo* (*31,32*). Potency can be improved by strategically located hydroxyls (*31*), and duration of action can be increased with alkyl ether derivatives (*17,18*). The most interesting observation, however, is the finding that a strategically located alkylaminoethoxy side chain can produce either agonist or antagonist actions. The principle is exemplified by the *cis* and *trans* geometric isomers of tamoxifen or clomiphene. Tamoxifen and enclomiphene (the *trans* isomers) are both partial estrogen agonists in the immature rat with antiestrogenic properties (*93–95*). In contrast, the *cis* isomers, ICI 47,699 (Fig. 15) and zuclomiphene, are both estrogens (*93–95*). These types of observations have been used to develop models to describe estrogen and antiestrogen action in the rat (*94,96*). The importance of a strategically located alkylaminoethoxy side chain for antagonist action has recently been illustrated (*97*) with derivatives of the estrogen cyclofenyl (Fig. 15). Removal of the acetyl groups and substitution of one phenol with a pyrrolidinoethoxy side chain produces an estrogen antagonist (*97*).

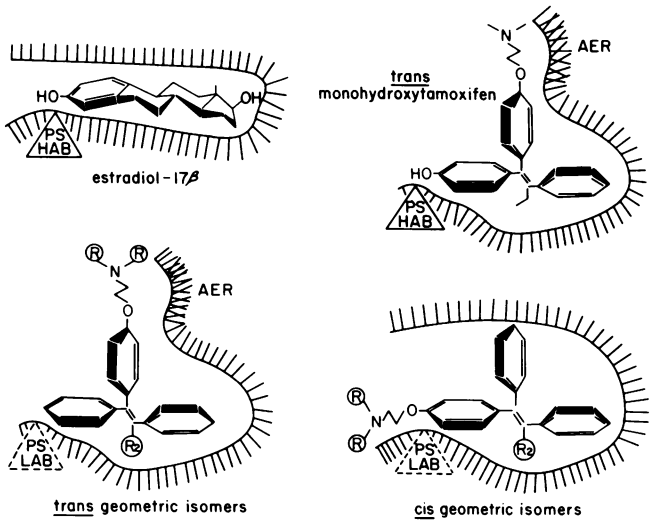


FIGURE 16. Hypothetical model for estrogen and antiestrogen binding to the estrogen receptor. Estradiol-17β is anchored at a phenolic site (PS) with high affinity binding (HAB). Compounds without a phenolic hydroxyl or without the correct stilbene-like structure would have low affinity binding (LAB). The *trans* and *cis* geometric isomers refer to: (*A*) tamoxifen (R = CH_3, R_2 = C_2H_5) and enclomiphene (R = C_2H_5, R_2 = Cl); (*B*) ICI 47,699 (R = CH_3, R_2 = C_2H_5) and zuclomiphene (R = C_2H_5, R_2 = Cl). An antiestrogenic ligand can bind to the receptor site so that the alkylaminoethoxy side chain can interact with a hypothetical antiestrogen region (AER) on the protein.

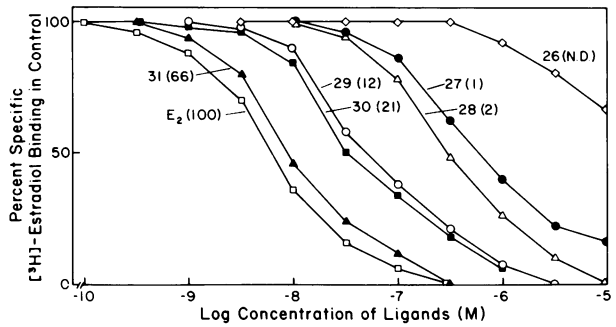


FIGURE 17. Inhibition of the binding of [^{3}H]estradiol to rat uterine estrogen receptors *in vitro* by different substituted triphenylethylenes desccribed in Fig. 15. The assay was conducted at 4°C and incubation was for 18 hr. The RBAs in parentheses are calculated as [(M) concn E_2 for 50% inhibition/(M) concn competitor for 50% inhibition] × 100.

Structure-Activity Relationships *in Vitro*: Receptor Binding Models

We have described a hypothetical model of the ligand interaction with the estrogen receptor. The model is based upon the structural features that are necessary to initiate or to inhibit prolactin synthesis in immature rat pituitary cells in primary culture. The simple interaction of estradiol-17β with a hypothetical binding site on the estrogen receptor has been taken as a starting point to evolve a model for drug–receptor interaction that results in the stimulation or inhibition of prolactin synthesis (Fig. 16). The foregoing arguments apply for the simple phenolic stilbene diethylstilbestrol.

We propose that the essentially planar estrogenic ligand first binds to the receptor via the phenolic group which produces a high affinity interaction. This initial point of attachment guides the rest of the molecule into the binding site. The ligand is then locked into the receptor and the tertiary changes that occur during this process activates the receptor complex. The antiestrogens, tamoxifen and 4-hydroxytamoxifen, completely inhibit estradiol-stimulated prolactin synthesis with potencies consistent with their RBAs for the estrogen receptor (*51*). It is interesting to point out that these antiestrogens are partial estrogens (partial agonists) *in vivo* (*98*) but *in vitro* there is no stimulation of prolactin synthesis. Overall, we have demonstrated that the inhibition of estradiol-stimulated prolactin synthesis by antiestrogens is competitive and reversible with estradiol, an effect that strongly suggests a mechanism via the estrogen receptor (*51*).

Introduction of a phenolic hydroxyl into tamoxifen to produce 4-hydroxytamoxifen increases receptor affinity and antiestrogenic activity (*53*). 4-Hydroxytamoxifen can bind with high affinity at the phenolic site on the receptor so that the phenyl ring containing the aminoethoxy side chain will extend away from the binding site to interact with an "antiestrogenic region" on the receptor (Fig. 16). This prevents the tertiary changes necessary for receptor activation so the complex is unable to initiate estrogenic events.

Among the triphenylethylenes, compounds that have *cis* and *trans* geometric isomers are extremely important for the development of a ligand receptor model because the isomeric molecules encompass both estrogenic and antiestrogenic properties. Although a geometric requirement for estrogenic activity has been stressed previously (*93*), these data derived from studies *in vivo* cannot exclude the possibility that the *cis* isomers (zuclomiphene or ICI 47,699) are preferentially metabolized to estrogens in the liver before binding in the target tissue. Zuclomiphene and ICI 47,699 are both estrogens *in vitro* (*44*).

To describe the interaction of the geometric isomers with the estrogen receptor, the *trans* stilbene-like structure of tamoxifen and enclomiphene could sit loosely at the binding site, with low affinity, so that the phenyl ring substituted with the *p*-alkylaminoethoxy side chain is projected away from the binding site (Fig. 16). The estrogenic ligands, zuclomiphene and ICI 47,699, with their low affinity for the receptor, can create a *trans* stilbene-like structure with the *para*-substituted phenyl ring. In this binding state, the aminoethoxy side chain would lie next to the phenolic site of the receptor with a weak interaction with the ether oxygen (Fig. 16). There would be no interaction of the side chain with the antiestrogenic region, and as a result, no inhibition of estrogen action.

We have probed this model in a study of acetylated triphenylbutenes (Fig. 15). For convenience the rings are labeled A, B and C. The RBAs of the compounds

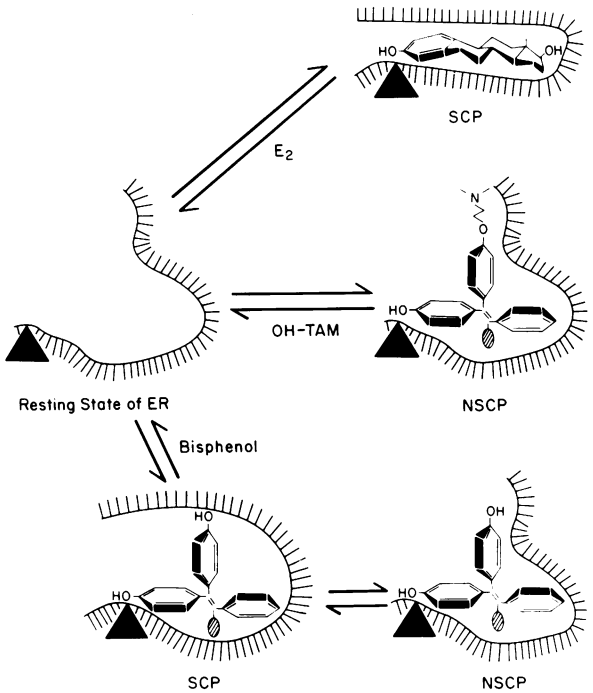


FIGURE 18. Adaptation of Belleau's macromolecular perturbation theory to describe the interaction of agonists, antagonists and partial agonists with the estrogen receptor (ER). The phenol groups on the ligand interact with the phenolic site (closed triangle) on the ER and produce a high affinity interaction if the geometry of the ligand is correct. E_2 (agonist) induces a specific conformational perturbation (SCP) whereas 4-hydroxytamoxifen (OHTAM antagonist) only induces a nonspecific conformational perturbation (NSCP). Bisphenol (partial agonist) produces a mixture of SCP and NSCP in the ER.

are shown in Figure 17. The unsubstituted triphenylbutene (compound 26) has the lowest affinity for the receptor but substitutions in the A and C rings dramatically improves affinity. Compound 30 with the acetyl in the equivalent position of the 3-phenolic hydroxyl of estradiol was the most active estrogen and the other compounds without substitutions in ring B were all estrogenic *in vitro* with potency directly related to their RBAs. Compound 31 has a high binding affinity for the receptor but is completely antiestrogenic *in vitro*. Clearly the aminoethoxy side chain is not the only substitution in ring B that will prevent estrogen action. Cyclofenyl falls into the same category *in vitro* (unpublished observation), but the cyclohexane ring demonstrates that the stilbene structure is not essential for effective receptor interaction. Of interest, however, is the observation that the removal of the acetyl groups to give bisphenolic compounds, produces partial agonists *in vitro*, i.e., compounds that can partially stimulate estrogen action. However, co-administration with estradiol causes a decrease in the full estrogen response. We have explained these observations on the basis of Belleau's macromolecular perturbation theory (*99*). According to Belleau's hypothesis, an agonist binds to the receptor and produces a specific conformational perturbation (SCP) and, as a result, the complex has an intrinsic efficacy of 1. An antagonist binds to the receptor to produce a nonspecific conformational perturbation (NSCP) and a complex with an intrinsic efficacy of zero. Between these extremes, a partial agonist binds to the receptor and produces an equilibrium mixture of agonist and antagonist receptor complexes. Applying these definitions to the estrogen receptor (Fig. 18), estradiol (agonist) binds with high affinity to the resting receptor and induces an SCP that results in the ligand being locked into the binding site. 4-Hydroxytamoxifen (antagonist) wedges into the resting receptor and only produces an NSCP. The bisphenol (partial agonist) interacts at the ligand binding site, but while some of the receptors can be induced to lock the ligand into the protein, other ligand interactions are only able to induce an NSCP in the complex.

Obviously further structure–activity relationship studies are required to consolidate these hypothetical views of ligand binding to the estrogen receptor binding site. However, other approaches can be taken to probe the estrogen receptor binding sites. Monoclonal and polyclonal antibodies to the estrogen receptor can bind to different sites on the protein. One monoclonal antibody, D547, will react with both estrogen and antiestrogen receptor complexes (*100*). Although the antibody cannot discriminate between agonist and antagonist receptor complexes, it can be used as a tool to examine their fate within target tissues. In contrast, we have recently described (*101*) the effect of a polyclonal antibody to the estrogen receptor that can distinguish between the binding of estrogens and antiestrogens. The final proofs of the differential binding of estrogens and antiestrogens to the estrogen receptor must come with the X-ray crystallography of purified estrogen and antiestrogen receptor complexes. This approach will almost certainly be feasible during the next decade. Mastery of techniques to clone the gene for the estrogen receptor will permit the production of "industrial" quantities of the protein for a biophysical investigation of drug–receptor interactions.

Summary of Structure–Activity Relationships

The structural requirements for estrogen action are different *in vivo* and *in vitro*. A general molecular skeleton for estrogen receptor binding compounds to produce effects in assays *in vitro* is shown in Figure 19.

The correct dimensions are necessary for an estrogenic ligand to occupy the receptor binding site.

A strategically located phenolic hydroxyl, not impaired by alkyl substitutions in the *ortho* position, is necessary for high affinity binding to the receptor and potent estrogenic activity *in vivo*.

Alkyl substitution of the 3′-phenolic hydroxyl of an estrogenic compound produces low affinity binding to the estrogen receptor but metabolic activation can occur

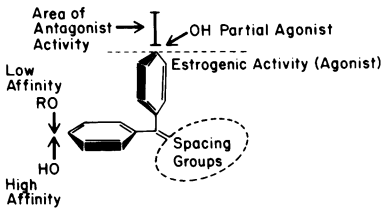


FIGURE 19. Basic structural features required by a compound to produce estrogenic, partial estrogenic or antiestrogenic actions *in vitro*. The potency of the compound is determined by a strategically located phenolic group on a ring equivalent to the A ring of the estrane steroid nucleus. Substitution of the phenol reduces affinity for the estrogen receptor and consequently the potency of the compound.

in vivo to produce potent estrogen action (mestranol to ethinyl estradiol).

Alkoxytriphenylethylenes (DBE, TACE) have a long duration of action because of a high solubility in body fat. There is metabolic activation to the phenolic derivatives.

A bisphenolic compound can occupy the receptor and produce estrogen action *in vivo* (the metabolite of methoxychlor, cyclofenyl). A stilbene-like structure is not required, only a spacing group to occupy the binding site.

A bisphenolic compound (with correct spacing groups) has a high affinity for the estrogen receptor but partial agonist activity *in vitro*.

Extending a side chain (alkylaminoethoxy or acetyl) away from the binding site in a bisphenolic compound produces a binding ligand with antiestrogenic properties *in vitro*.

Nonsteroidal antiestrogens based upon triphenylethylene with an alkylaminoethoxy side chain (tamoxifen) are complete antagonists *in vitro* but partial agonists *in vivo*.

Acetylated antiestrogens *in vitro* (cyclofenyl) are deacetylated *in vivo* to produce full estrogen action; bisphenolic triphenylethylenes that are partial agonists *in vitro* are full agonists *in vivo*.

Several of the general structural features that are required for estrogenic activity may aid the investigation of novel compounds in the future.

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